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Title: Calcitonin-gene related peptide and disease activity in cluster headache

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38 interpretation, drafting the manuscript.

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50 intellectual content.

51 Rigmor Jensen: Study concept and design, interpretation of study result, supervision

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77

78 **Abstract**

79 **Objective:** To investigate the role of calcitonin gene-related peptide (CGRP), pituitary adenylate
80 cyclase-activating polypeptide-38 (PACAP38) and vasoactive intestinal polypeptide (VIP) in
81 cluster headache (CH), we measured these vasoactive peptides interictally and during
82 experimentally induced CH attacks.

83 **Methods:** We included patients with episodic CH in an active phase (n=9), episodic CH patients in
84 remission (n=9) and in patients with chronic CH (n=13). Cluster headache attacks were induced by
85 infusion of CGRP (1.5µg/min) in a randomized, double-blind, placebo controlled, two-way cross-
86 over study. At baseline we collected interictal blood samples from all patients and during 11 CGRP-
87 induced CH attacks.

88 **Results:** At baseline, episodic CH patients in remission had higher plasma levels of CGRP, $100.6 \pm$
89 36.3 pmol/l, compared to chronic CH patients, 65.9 ± 30.5 pmol/l, ($p=0.011$). Episodic CH patients
90 in active phase had higher PACAP38 levels, 4.0 ± 0.8 pmol/l, compared to chronic CH patients, 3.3
91 ± 0.7 pmol/l, ($p=0.033$). Baseline levels of VIP did not differ between CH groups. We found no
92 attack-related increase in CGRP, PACAP38 or VIP levels during CGRP-induced CH attacks.

93 **Conclusions:** This study suggests that CH disease activity is associated with alterations of CGRP
94 expression. Future studies should investigate the potential of using CGRP measurements in
95 monitoring of disease state and predicting response to preventive treatments including response to
96 anti-CGRP monoclonal antibodies.

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101 **Introduction**

102 The hallmark of cluster headache (CH) is periodicity and prominent cranial autonomic symptoms
103 (CAS) ¹. Most patients report periodicity by experiencing episodic CH with month long attack
104 periods separated by remission periods ². To what extent mechanisms underlying CAS contribute to
105 initiation of CH attacks is still not fully elucidated. The trigemino-autonomic reflex activation is
106 associated with release of sensory and parasympathetic neuropeptides ³ such as calcitonin gene-
107 related peptide (CGRP), pituitary adenylate cyclase-activating polypeptide-38 (PACAP-38) and
108 vasoactive intestinal polypeptide (VIP). Studies investigating plasma levels of these neuropeptides
109 in CH remain, however, scarce and conflicting ⁴⁻⁸. Elevated plasma CGRP, VIP and PACAP38
110 have been reported during spontaneous and glyceryl trinitrate (GTN) provoked CH attacks ^{5-7,9}. The
111 diverging methodologies of these studies, however, make it difficult to compare findings across
112 studies. To date, no studies have investigated the role of these vasoactive peptides in chronic CH
113 patients. In addition, it is unknown whether plasma levels correlate to disease periodicity. As CH is
114 unpredictable and short lasting, investigation of patients during spontaneous attacks can be
115 immensely difficult. This challenge can be overcome by studying provoked CH attacks in a
116 controlled setting ⁵. Recently, we demonstrated that CGRP infusion could provoke CH attacks in
117 patients in an active disease state (episodic CH in active phase and chronic CH patients), but not in
118 patients during remission ¹⁰.

119 We hypothesized that baseline levels of CGRP, VIP and PACAP38 would be elevated in
120 episodic and chronic CH patients in an active disease state compared to patients in remission.
121 Furthermore, we hypothesized that CGRP-induced CH attacks would cause a further increase in
122 plasma levels of neuropeptides. To test these hypotheses, we investigated plasma CGRP, VIP and
123 PACAP38 at baseline and during CGRP induced CH attacks. In addition, we compared the baseline

concentration of neuropeptides in CH patients with historical data on migraine patients and healthy controls.

Materials and Methods

Patients were eligible for inclusion if they were aged 18 – 65 years and had a verified diagnosis of episodic or chronic cluster headache as defined by the International Headache Society classification (Headache Classification Committee of the International Headache Society (ICHD-3 beta), 2013)

¹¹. We recruited participants from the outpatient clinic at the Danish Headache Center (Rigshospitalet-Glostrup) in the period from December 2015 to April 2017. The present study is a predefined part of a larger parent protocol (protocol H-15006836, clinicaltrials.gov identifier NCT02466334). The first part of the study investigated the ability of CGRP to induce cluster headache like attacks and has previously been described in detail ¹⁰. Patients were eligible for inclusion in the study if they were in active disease phase, defined as occurrence of typical CH attacks within the last 30 days; or in remission, defined as attack-free for at least 30 days. Episodic patients could participate in remission and in active disease phase. According to the ICHD-3 beta criteria, chronic patients did not have attack-free periods exceeding 30 days in the last 12 months. Exclusion criteria included any other type of headache (apart from episodic tension-type headache \leq 5 days per month), any previous serious somatic or psychiatric condition, pregnant or nursing women, drug misuse or daily intake of medication other than preventive treatment for CH. All patients underwent a full medical examination and in women of childbearing age pregnancy testing was conducted prior to participation.

The study was approved by the Regional Committee on Health Research Ethics of the Capital Region (H-15006836) and was conducted in accordance with Helsinki II Declaration of 1964, with later revisions. The study was registered at clinicaltrials.gov (identifier NCT02466334) and

148 approved by the Danish Data Protection Agency. All patients received oral and written information
149 about the study and were given time for consideration before giving their written consent to
150 participate.

151 In the present study we conducted post hoc analyses which included previously published data on
152 migraine patients and healthy volunteers ¹² (ClinicalTrials.gov identifier NCT01841827). All
153 samples from previous data were collected in an inter-ictal state, defined as the participant being
154 completely headache- and analgesic free for a minimum of 48-hours prior to sampling. Samples
155 were analyzed by the same assays in the same laboratory as the current study ¹².

156 *Design and experimental protocol*

157 The study was conducted as a randomized, double-blind, placebo controlled, two-way cross-over
158 study. All patients were randomly allocated to receive a continuous infusion (Braun Perfusor,
159 Melsungen, Germany) with either 30 µg CGRP (1.5µg/min) (Calbiochem® and PolyPeptide group)
160 or placebo (saline) over 20 min on two separate days. CGRP and placebo were prepared in identical
161 vials and randomized by the regional central pharmacy. Allocation was balanced to ensure
162 approximately even numbers of participants receiving CGRP first and placebo last or vice versa.
163 The randomization code remained in the hospital during the study and was unavailable to
164 investigators until study completion.

165 On both experimental days, patients with episodic (active phase) and chronic cluster headache
166 reported themselves to the clinic when they were headache/attack free for at least 3 and 8 hours,
167 respectively. An 8-hour headache-free-interval prior to provocation was initially set for both episodic
168 patients in cluster and chronic cluster patients, but due to feasibility concerns, a revised 3-hour
169 headache-free-interval was set in order to include episodic patients with a high mid-cluster attack
170 burden.

171 All participants were asked to retrospectively estimate their attack frequency in the preceding 30
 172 days. Patients were placed in a supine position and a venous catheter (Venflon®) inserted in the
 173 cubital vein on the right or left arm for CGRP infusion and drawing of blood samples. Patients were
 174 at rest for 15 min before obtaining baseline status. Blood for analysis of CGRP, PACAP38 and VIP
 175 was drawn at fixed time points: At baseline (T0), post infusion (T20), 10 min (T30) and 70 min post
 176 infusion (T90). If the patient developed a CH-like attack during the observations period, blood was
 177 drawn at the onset phase of attack (Ta0), after 15 min (Ta15) and at 30 min after the start of the
 178 attack (Ta30).

179 *Blood collection and processing*

180 Blood was drawn through the venous catheter and connector using two 20 ml syringes. For blood
 181 sampling the first 5 ml were discarded and after the procedure the catheter was flushed with saline.
 182 The blood was thereafter transferred into different tubes: precooled lithium heparin tubes containing
 183 aprotinin (Trasylol®) for VIP; precooled EDTA tubes with aprotinin for PACAP38 and standard
 184 EDTA tubes for CGRP. All tubes were inverted several times. The precooled tubes were stored in a
 185 cooling box (5°C) and the rest stored at room temperature for 20 min until centrifugation. The tubes
 186 were centrifuged together at 4°C at 1851g for 10 min. Plasma was thereafter transferred to
 187 polypropylene tubes (Greiner Cryo.s™) and stored at -25°C until analysis.

188 *Radioimmunoassay*

189 Plasma CGRP concentrations were measured with a fully evaluated radioimmunoassay for human
 190 CGRP, as described previously (Schifter, 1991)^{12,13}. The tracer was prepared by the method of
 191 Iodogeneral (Pierce, Rockford, IL, USA)¹⁴ by iodination of [Tyr0] α -CGRP (25-37) amide and
 192 purification by high liquid chromatography (HPLC). Samples, antibody and calibrators were
 193 incubated at 4°C for about 90 hours before addition of tracer and subsequent incubation for 48
 194 hours. Free and antibody-bound tracer was separated by Sac-Cel separation.

195 *Plasma concentration of PACAP38*

196 The concentration of PACAP38 in plasma was measured radioimmunochemically using antiserum
 197 733C-5 directed against the sequence PACAP28–38¹⁵. The antiserum which was used at a final
 198 titer of 1.2×10^5 in a total volume of 0.8 ml/tube does not cross-react with PACAP27, VIP or other
 199 structurally related peptides. Synthetic PACAP28–38 labeled to a specific radioactivity of 30
 200 Bq/mol with ¹²⁵I by the iodogen method was used as tracer and synthetic human PACAP38 was
 201 used as standard. The IC₅₀ value (the concentration of PACAP38 giving 50% displacement of the
 202 tracer) was 17 pmol/l and the intra-assay and inter-assay coefficient of variation values were 3.1 and
 203 10.1%, respectively.

204 Since PACAP38 in human plasma is bound to the protein ceruloplasmin¹⁶ the peptide was freed
 205 from ceruloplasmin before measurement by the following procedure: 1.2 ml of 1% trifluoroacetic
 206 acid was added to an equal volume of plasma from each subject and mixed thoroughly for 60 s.
 207 After incubation for 10 min in an ice-bath, the mixture was neutralized by addition of 15 µl of 5 M
 208 NaOH. Subsequently 2.5 ml of absolute ethanol was added. After thorough mixing, followed by
 209 centrifugation at 1500 g for 20 min at 4 °C, the supernatant was decanted and dried under vacuum.
 210 The dried product was reconstituted to its original volume with assay buffer for assay.

211 *Plasma concentration of VIP*

212 The concentration of VIP in plasma, after extraction with absolute ethanol, was measured by the
 213 VIP radioimmunoassay using antiserum 5603–6 at a final titer of 1.2×10^6 in a total volume of 0.8
 214 ml/tube^{17,18}. This antiserum recognizes the mid- and C-terminal regions of the VIP molecule
 215 (sequence 11–24) and displays no cross-reactivity with other known gastrointestinal peptides or
 216 neuropeptides. The label has a specific radioactivity of 0.92 nCi/fmol (~34 Bq/fmol). The IC₅₀
 217 value (the concentration of VIP giving 50% displacement of label) was 24 pmol/l and the intra-
 218 assay and the inter-assay coefficient of variation values were 8.7 and 12.6%, respectively.

219 *Headache characteristics and vital signs*

220 From baseline (T-10 and T0) and throughout the entire experiment the following variables were
 221 recorded every 10 min: Headache intensity on a verbal rating scale (VRS) from 0 to 10 (0; no
 222 headache, 1; very mild headache, 10; worst imaginable headache); quality of pain (stabbing,
 223 throbbing, pulsating or resembling usual CH attack); headache localization and accompanying
 224 symptoms, these including CAS. In addition, we recorded blood pressure and heart rate. Symptoms
 225 experienced outside recording intervals were documented separately.

226 *Statistical analysis*

227 All absolute values are presented as mean \pm standard deviation. The primary endpoints were: 1)
 228 Differences of biochemical variables (CGRP, PACAP38 and VIP) in between groups (episodic CH
 229 patients in active phase, episodic CH patients in remission and chronic CH patients) at baseline; 2)
 230 Differences over time in plasma concentrations of biochemical variables between patients
 231 developing an attack and those who did not. 3) Differences in plasma concentrations over time of
 232 biochemical variables between active and placebo days.

233 Distribution of demographical data was tested using the D'Agostino and Pearson normality test and
 234 group comparisons of demographical data were subsequently analyzed using parametric statistics.
 235 Evaluation of baseline variables was done using a generalized linear model with repeated
 236 measurements.

237 To analyze for an effect of CGRP infusion on biochemical variables we used repeated
 238 measurements analysis with random effect of subjects, attacks and further of subjects' times day. In
 239 this way we allow for correlation between measurements on the same individual, and additional
 240 correlation between measurements in the same individual on the same day. The measurement taken
 241 at time zero was used as baseline variable in the repeated measurements model. For each of the

responses VIP, PACAP and CGRP we checked model assumptions and transformed the response variables as appropriate to meet model requirements. Correlations between baseline levels and time since last attack, were calculated using Pearson correlation test.

We used GraphPad Prism 7.02, SAS Enterprise and R 3.4.3 for statistical analyses. All p-values were two-sided and considered significant if <0.05 .

Data availability

The data supporting the findings of this study are not publicly available, but will be shared, in an anonymized form, by request from any qualified investigator.

Results

In total 31 patients (26 men and 5 women) completed the study (Fig. 1). The mean age was 37 years, (range 19 – 59). Nine patients reported episodic CH in active phase (6 men, 3 women; mean age 32, range 19 – 56 years), 9 episodic in remission (all men; mean age 32, range 22 – 43 years), and 13 chronic CH (10 men, 3 women; mean age 42, range 26 – 59 years). Clinical data on patients are shown in table 1. Episodic patients in remission reported remission on average for 6.6 (range 1.3–18.0) months prior to participation in the study. At baseline, blood samples were collected in all 31 patients for VIP and CGRP, but samples from one patient were lost for PACAP38. CGRP infusion induced a CH-like attack in 16 out of 31 patients, of these non in episodic patients in remission. We collected blood samples during 11 out of 16 CH attacks and all attack samples were collected prior to abortive treatment. In the remaining 5 attacks, symptoms subsided before we had the chance to engage attack sampling protocol. All provoked attacks were unilateral, located in the periorbital region and were accompanied by CAS and/or restlessness. The median severity of provoked attacks was 10 (IQR 4 – 10, range 1 – 10) and median number of accompanying symptoms was 4 (IQR 1.5 – 5, range 1 – 8). Characteristics of 11 provoked attacks are listed in

265 table 2. The concentration of CGRP, PACAP38 and VIP were above the detection limit in all blood
 266 samples.

267 *CGRP*

268 We found significantly higher baseline plasma CGRP in episodic patients in remission, $100.6 \pm$
 269 36.3 pmol/l, compared to chronic patients, 65.9 ± 30.5 pmol/l, ($p=0.011$) (Fig. 2, table 3). The
 270 repeated measurements analysis showed no independent increase of plasma CGRP in patients who
 271 reported a CH-like attack after provocation with CGRP ($p=0.36$). After CGRP infusion plasma
 272 levels of CGRP increased significantly, 574.3 ± 296.4 pmol/l, compared to baseline, 81.7 ± 33.4
 273 pmol/l, ($p<0.0001$). We found no changes in CGRP levels after placebo infusion compared to
 274 baseline ($p=0.43$).

275 *PACAP38*

276 We found significantly higher baseline PACAP38 levels in episodic patients in active phase, $4.0 \pm$
 277 0.8 pmol/l, compared to chronic patients, 3.3 ± 0.7 pmol/l, ($p=0.033$) (table 3). CGRP induced CH
 278 attacks were not associated with changes in plasma PACAP38 ($p=0.29$). Compared to baseline,
 279 plasma levels of PACAP38 remained unchanged after CGRP ($p=0.66$) and placebo ($p=0.57$)
 280 infusion.

281 *VIP*

282 We found no differences in baseline plasma levels of VIP between CH groups ($p > 0.05$) (table 3).
 283 The repeated measurements analyses revealed a significant decrease in patients who reported a
 284 provoked attack ($p=0.013$). Infusion of CGRP caused a significant increase in plasma VIP
 285 compared to baseline ($p<0.001$), but not after placebo ($p=0.53$).

286 *Post hoc analysis*

We compared five groups: Episodic patients in active phase; episodic patients in remission; chronic patients; migraine without aura patients and healthy controls (Fig. 2). The analysis revealed that episodic patients in bout ($p<0.001$), patients in remission ($p<0.001$) and chronic patients ($p=0.020$) had higher baseline levels of CGRP compared to healthy controls. Furthermore, patients in active phase ($p<0.001$) and in remission ($p<0.001$) had higher CGRP levels compared to migraine patients. We found no differences in PACAP38 and VIP levels between CH patients, migraine patients and healthy controls.

In patients in an active disease state we found no difference in baseline CGRP, PACAP38 nor VIP levels in patients on prophylactic treatment vs. those without ($p>0.05$). There was no correlation between hours since last attack, nor 30-day attack burden and baseline level of CGRP, PACAP38 nor VIP ($p>0.05$).

Discussion

The main finding of the present study was that CH patients in remission had higher baseline levels of CGRP, but not VIP or PACAP38, compared to chronic CH patients. Furthermore, CGRP-induced CH attacks were not associated with elevated levels of CGRP, VIP or PACAP38. In addition, CH-patients irrespective of disease state had higher plasma levels of CGRP compared to migraine patients and healthy controls.

A novel finding of the present study was that chronic CH patients had lower plasma CGRP compared to CH patients in remission. This suggests that plasma levels of CGRP may fluctuate with disease activity. The results are very interesting when viewed in the light of recent press releases on the preventive effect of the anti-CGRP monoclonal antibodies in episodic patients in active period, but not in patients with chronic CH. Interestingly, poor treatment response in chronic CH is reported across different treatment modalities indicating basic pathophysiological differences

311 in between phenotypes^{19,20}. The question is why chronic CH patients had lower CGRP levels than
 312 CH patients in remission and episodic CH patients in active phase? Furthermore, how the regulation
 313 of CGRP expression and release aligns with our data is difficult to reconcile but CGRP expression
 314 is regulated in many ways. Animal experiments reported local elevated CGRP levels after nerve
 315 damage, nerve regeneration and inflammatory response²¹. CGRP is expressed and released from C
 316 fibers and its receptors are present in A delta fibers²². In man, application of capsaicin to the nasal
 317 mucosa led to immediate release of CGRP in saliva and plasma⁸. It is possible that secreted CGRP
 318 may act in an autocrine fashion to further increase CGRP release in a positive feedback loop, a
 319 mechanism possibly implicated in peripheral sensitization²³. In rats, a single subcutaneous
 320 capsaicin injection in the hind-paw depleted CGRP levels in the skin and sciatic nerve after 8 and
 321 10 days^{24,25}. Interestingly, repeated capsaicin applications to the nasal mucosa resulted in
 322 desensitization and time-dependent recovery of responses²⁶. In addition, intradermal capsaicin
 323 injections produced a steady increase of CGRP levels in the first sampling period but failed to reach
 324 significance in the second session²⁷. Thus, capsaicin-induced desensitization of sensory afferents
 325 might lead to depletion of neuropeptide release from afferents or decreased activity of transient
 326 receptor potential vanilloid 1 channels^{28,29}. Taken together these data suggest that chronic CH
 327 patients may exhibit low plasma CGRP due to depletion of CGRP from trigeminal afferents.
 328 Whether a CH attack represents a comparable stimulus to capsaicin is unknown, but it is possible
 329 that endogenous processes influence CGRP expression. Several factors might have influenced our
 330 data. We tested for possible influence of a recent attack and attack burden (frequency) and found
 331 that CGRP levels were not associated to the most recent attack or to attack burden in the 30 days
 332 preceding baseline sampling. Exogenous factors such use of preventive and abortive treatments may
 333 theoretically influence CGRP expression. One study reported that treatment with corticosteroids can
 334 reduce CGRP levels in episodic CH patients⁴. In the present study 61.5% of chronic CH patients

335 took preventive treatments compared to 44.4 % of episodic CH patients, but an exploratory analysis
336 revealed no difference in baseline levels. Preclinical studies reported that application of 5-HT₁
337 receptor agonist decreased the synthesis of CGRP in trigeminal ganglion³⁰ and that 7 day infusion
338 of sumatriptan infusion upregulated CGRP expression in trigeminal dural afferents³¹. In the present
339 study, we did not record the patients use of triptans prior to baseline sampling, but in future studies
340 investigating CGRP in CH, this should be taken into consideration.

341 In the post hoc analyses we found that episodic patients in active phase and in remission had
342 higher baseline levels of CGRP than episodic migraine patients and healthy controls. Although data
343 from migraine patients and healthy controls derived is historical and thus should be interpreted with
344 caution, it is an interesting observation. One study reported that chronic migraine patients had
345 higher CGRP levels than episodic migraine patients, healthy controls or episodic CH patients in
346 remission³². This study also reported no difference between patients with episodic migraine and
347 CH patients³². As different assays have been used across studies in migraine and CH it is
348 impossible to compare results directly and further hypothesis-based studies are necessary to address
349 possible difference in CGRP levels between cluster headache and migraine.

350 Collectively, our data suggest that CGRP may be altered in CH, but the findings should be
351 reproduced in a larger cohort of CH patients, ideally in prospective studies investigating changes in
352 CGRP over time and disease state.

353 In the present study chronic CH patients had lower baseline levels of PACAP38 compared to
354 episodic patients in bout, but similar levels compared to CH patients in remission. In line with our
355 CGRP findings in chronic CH patients, this suggests possible pathophysiological differences
356 between disease states. Another important finding was that CGRP-induced CH attacks were not
357 associated with alterations in plasma PACAP38 or VIP. These data are in contrast to previous
358 studies, where VIP⁷ and PACAP38⁹ were reported elevated during spontaneous attacks. In the

359 current study chronic patients had an average longer disease duration compared to episodic patients,
 360 which might be an influencing factor. A migraine study reported that baseline levels of PACAP38
 361 were negatively correlated with disease duration ³³, suggesting that longer disease duration or
 362 transition to the chronic phase alters the regulation of this peptide. In support, repeated chemical
 363 stimulation of dura surrounding the superior sagittal sinus decreased PACAP38 levels in the TG in
 364 rats ³⁴. Both PACAP38 and VIP may be considered as markers of parasympathetic activation.
 365 Higher PACAP38 levels in episodic CH patients in active phase compared to chronic patients could
 366 theoretically reflect marked parasympathetic activation, but as VIP was unchanged, this
 367 interpretation remains speculative. To notice, we found no correlation between hours since last
 368 attack and baseline VIP or PACAP38 levels, as would be expected given the short half-lives (min)
 369 of VIP and PACAP38 ³⁵. Infusion of CGRP induced an increase in VIP, but not PACAP38, and
 370 development of an attack was associated with a decrease in VIP. As VIP levels were highest
 371 immediately following infusion and attacks occurred on average 34 min after onset of infusion, the
 372 observed attack-associated decrease in VIP was likely to coincide with the natural fall in VIP.
 373 Interestingly, our previous study using the same assay also found elevated VIP after CGRP infusion
 374 in migraine patients ¹². These findings suggest that CGRP infusion is associated with transient
 375 elevation of plasma VIP, but it does not seem to be associated with attack development.
 376 Collectively, CH disease activity was not associated with elevation of PACAP38 and VIP.
 377 Furthermore, CGRP induced CH attack did not increase plasma VIP and PACAP38, which is in
 378 contrast to previous studies ^{7,9}. The inconsistency of results across studies is likely attributed to use
 379 of different assays ³⁵.

380 We collected blood from the antecubital vein, and not from the external jugular vein.
 381 One might argue that plasma levels should be collected in the cranial outflow. In migraine patients,
 382 elevated plasma CGRP was reported elevated in peripheral blood ictally ³⁶ and interictally ³⁷. One

383 study in migraine patients reported no changes in plasma CGRP during attacks. To date, no studies
384 have compared plasma PACAP38 and VIP between the two sampling sites, but elevated levels of
385 these neuropeptides were reported in peripheral blood during and outside of migraine attacks^{9,33}.
386 We acknowledge relatively small sample size in the present study. However, we obtained two sets
387 of samples for baseline-comparisons (in total 62 samples) and performed a robust statistical
388 analysis. We collected “attack samples” in 11 patients. Previous studies reported plasma alterations
389 during and spontaneous attacks on average of 14 patients^{5–7,9}. Thus, the present sample size should
390 be sufficient to detect possible attack related changes in neuropeptides. As blood samples were not
391 drawn in all provoked attack and element of selection bias might affect attack results. However, as
392 these results were negative, the concern for this bias seems less important. With regards to
393 sensitivity and specificity of CGRP assay used in the current study, we measured a robust elevation
394 of CGRP after CGRP infusion and importantly no concomitant increase in PACAP38, which is
395 structurally similar.

396 The present study demonstrated that CH disease activity might be associated with
397 alterations of CGRP expression, possibly PACAP38 but not VIP expression. Future studies should
398 investigate the potential of using CGRP measurements in monitoring of disease state and predicting
399 response to preventive treatments including response to anti-CGRP monoclonal antibodies.

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405

Article highlights:

- The present study demonstrated that cluster headache disease activity is associated with alterations of CGRP expression, possibly PACAP38 but not VIP expression
- Our results indicate that there are basic pathophysiological differences between episodic and chronic cluster headache patients
- The observed lower CGRP levels in chronic cluster headache patients at baseline might offer an explanation to why anti-CGRP monoclonal antibodies have proven effective in episodic, but not in chronic cluster headache patients

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522 **Figure 1.** Flow chart of recruitment and inclusion of patients.

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525 **Figure 2. Baseline levels of CGRP, VIP and PACAP38 presented as means and SD.**

526 Dashed lines divide present data from historical data. Significant findings according to primary
527 endpoints marked. Significant findings in post hoc analyses comparing CH patients to data from
528 historical data: CGRP, eCHr and eCHa > HC, $p < 0.001$; eCHr > MO, $p < 0.001$; eCHa > MO,
529 $p < 0.001$; cCH > HC, $p = 0.020$. No differences regarding VIP and PACAP38 levels between CH
530 patients and MO or HC patients were found.

531 cCH = chronic cluster headache; eCHa = episodic cluster headache patient in active phase; eCHr =
532 episodic cluster headache in remission; MO = Migraine without aura; HC = Healthy controls;
533 CGRP = Calcitonin gene-related peptide; PACAP38 = pituitary adenylate cyclase-activating
534 polypeptide-38; VIP = Vasoactive intestinal peptide.

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536

Table 1. Clinical data on patients with episodic cluster headache in active phase (eCHa), remission (eCHr) and chronic cluster headache (cCH).

eCHa = episodic cluster headache patient in active phase; eCHr = episodic cluster headache patients in remission; cCH = chronic cluster headache patients; SD = standard deviation, W/M = Women/Men. *Treatments: Verapamil 400mg; Verapamil 560mg; Verapamil 400 mg; Recent blockade greater occipital nerve. ** Treatments: Verapamil 400mg; Verapamil 800mg. *** Treatments: Verapamil 440mg; Verapamil 480mg; Verapamil 100mg; Verapamil 400mg and Melatonin 8mg, Verapamil 240mg, Verapamil 600mg; Verapamil 240mg and Lithium 200mg; Sphenopalatine ganglion neurostimulation and melatonin 4mg.

557 **Table 2.** Clinical characteristics of provoked attacks in 11 patients.

558

559 Headache intensity: 0 – 10 Verbal response scale. Ta0: Attack onset, prior to acute therapy. Ta15

560 and Ta30: 15 and 30 min after attack onset respectively. Acute therapy: Suma = sumatriptan 6mg

561 sc; Oxy = oxygen 15L/min Optimask; SPG = The Pulsante SPG Microstimulator System; Dic =

562 Diclofenac 25mg sc. Accompanying symptoms: lac = lacrimation; pto = ptosis; mio = miosis; con =

563 nasal congestion; inj = conjunctival injection; swe = forehead and facial sweating; res =

564 restlessness; rhi = rhinorrhea; ede = eyelid edema.

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575 **Table 3.** Levels of CGRP, PACAP38 and VIP at baseline, presented in means \pm SD.

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577 eCH = episodic cluster headache; cCH = chronic cluster headache; CGRP = calcitonin gene-related
578 peptide; PACAP38 = pituitary adenylate cyclase-activating polypeptide-38; VIP = vasoactive
579 intestinal polypeptide

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